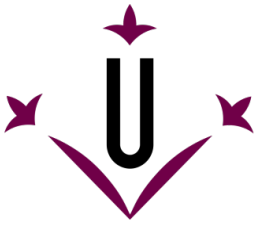

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Title

Smoke, pheromone, and kairomone olfactory receptor neurons in males and females of the pine sawyer *Monochamus galloprovincialis* (Olivier) (Coleoptera:Cerambycidae).

Authors

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Abstract

The response of antennal olfactory receptor neurons (ORNs) of *Monochamus galloprovincialis* to several odorants was tested using single sensillum electrophysiology. Behaviorally active pheromone, and kairomone (host and sympatric bark beetle pheromone) odors were tested alongside smoke compounds released by burnt wood that are potentially attractive to the insect. The antennae bore several types of sensilla. Two plate areas in the proximal and distal ends of each antennal segment were covered with basiconic sensilla that responded to the odor stimuli. Sensilla basiconica contained one or two cells of different spike amplitude. The 32 male and 38 female ORNs tested responded with excitations or inhibitions to the different plant odors. In general the response of male and female receptors was very similar so they were pooled to perform a cluster analysis on ORN responses. Six ORNs were clearly specialized in pheromone reception. Responses to kairomone and smoke odors were less specific than those of pheromone, but a group of 9 cells was clearly excited by smoke compounds (mainly eugenol and 4-methyl 2-methoxyphenol), a group of 8 cells was very responsive to α-pinene, β-pinene and *cis*-verbenol, and a group of 14 cells responded to a wider range of compounds. The rest of the cells (47%) were either non-responsive or slightly inhibited by smoke compounds. Dose-response curves were obtained for several compounds. Different compounds induced significantly different latencies and these appeared to be unrelated to their boiling point.

Key words

Single sensillum recording, pine wilt disease, olfaction, electrophysiology, attractant, detector

1. Introduction

Most long-horn beetle species are considered secondary forest pests, but those in the genus *Monochamus* Dejean (Coleoptera: Cerambycidae) have become economically important worldwide as they vector pine wood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer), (Linit and Akbulut, 2008) which causes pine wilt disease (PWD) in vulnerable pine species (Wingfield, 1982). *B. xylophilus* is endemic to North America but, beginning early last century, PWD was accidentally introduced in several countries with devastating environmental and economic consequences (Shing, 2008; Zhao et al., 2008). In Europe, PWD was reported for the first time in Portugal (Mota et al., 1999). It spread over most of the country, despite efforts to control it (Rodrigues, 2008), and it was detected in Spain for first time in 2008 (Espárrago, 2012). Four infection foci have been declared in Spain since then, and strong measures for eradication (Evans et al., 1996) are currently being implemented .

One of the most promising strategies in PWD management in Europe is to act on *M. galloprovincialis* (Olivier), the only known vector described for this disease in the continent (Sousa et al., 2001). In recently years, significant progress on the chemical ecology of *M. galloprovincialis* has been achieved with the aim of developing more effective monitoring and management tools (Pajares et al., 2004; Ibeas et al., 2007; Pajares et al., 2010; Álvarez et al., 2014). The genus *Monochamus* responds to host tree volatiles such as α-pinene or ethanol, as well as to pheromone components of pine-scolytids, such as ipsenol, ipsdienol, *cis*-verbenol and 2-methyl-3-buten-2-ol (Billings and Cameron, 1984; Billings, 1985; Allison et al., 2001, 2003; De Groot and Nott, 2004; Miller and Asaro, 2005). Some of these compounds have been described as

attractants of *M. galloprovincialis* in Europe (Pajares et al., 2004; Ibeas et al., 2007; 2008). In addition, male *M. galloprovincialis* release an aggregation pheromone (2-undecyloxy-1-ethanol) which attracts both males and females (Pajares et al., 2010). The potency of this pheromone increases with the addition of host and bark beetle kairomones (Pajares et al., 2010). The antenna of *M. galloprovincialis* responds to the aggregation pheromone and to bark beetle and host kairomones (Pajares et al., 2010). Up to now, a morphological and physiological study at the sensillum and olfactory receptor neuron (ORN) levels has not been carried out, being also very rare in cerambycids (Dyer and Seabrook, 1978; Barata et al., 2002) although functional specificity in olfactory receptor neurons has been found in other beetles (Bengtsson et al, 2009; Andersson et al, 2009).

The present paper reports studies of the different types of sensillae and their distribution along the antenna of *M. galloprovincialis*, and records the response of the olfactory receptor neurons (ORNs) housed within to volatiles in smoke, host-plant volatiles, pheromone components of pine scolytids and the aggregation pheromone of *M. galloprovincialis*, in order to gain a better understanding of the function and specificity of these receptors.

2. Materials and Methods

2.1 Insects

Logs containing *M. galloprovincialis* larvae were collected from fire-damaged trees in Valencia, Spain in the winter-spring of 2012 and were left in an outdoor cage until adult emergence during the following summer. Adults were stored individually in 1-l glass jars under a 15L:9D photoperiod and 15:22ºC temperature regime, and were provided with fresh pine twigs for at least 2 weeks before testing to ensure enough time to reach sexual maturity (Naves et al., 2006).

2.2 Scanning electron microscopy

Four antennae from each sex were cut and mounted on microscope holders with conductive double-side adhesive black tape. Preparations were air dried at 60ºC for 2 days, fixed with osmium tetroxide, dehydrated with acetone and then coated using a sputter coater (Balzers SCD 050, Leica Microsystems, Madrid, Spain), with 50 nm gold particles for 3 minutes from a distance of 50 mm, with a current of 45 mA and Argon as cooling gas. Samples were scanned using a Zeiss DSM940A microscope with 10 kV at 200X to 500X magnifications. A rough estimate of the relative distributions of different types of sensilla was obtained by counting the number of sensilla inside a 200 µm-side square placed in the proximal, medial and distal regions in the lateral side of flagella 1, 3, 6 and distal. Length and basal widths of all types of sensilla contained in the sample area (N = 10 when feasible) were measured.

2.3. Electrophysiological recordings

Whole insects were immobilized and fixed on a microscope slide with parafilm and double-sided adhesive tape (Fig. S1). Antennae were attached to the microscope slide using double-sided adhesive tape and immobilized with a strip of dental wax over the first flagellum and with several small pieces of tape throughout its length, but leaving proximal, medial and distal areas of each segment exposed. The slide was attached to a magnet (4x2x1 cm) to fix it to the metal antivibration table (63–511, TMC Ametek, USA). The electrodes consisted of electrolytically (20% KNO_2) sharpened tungsten microelectrodes (0.125 mm diameter, 99.98% purity, Advent Research Materials Ltd, England). The reference electrode was inserted into the first flagellum while the recording electrode was placed in the base of randomly chosen sensillae on the opposite antenna, with the help of a manual micromanipulator (NMN-25, Narishige, Japan) under a stereo-microscope (objective 2 x, oculars 25 x, zoom range 0.8-12.5, Leica Microsystems, Madrid, Spain). The recorded signal was preamplified (PR-05, Syntech, Germany), filtered and digitized (low pass = 200 Hz, high pass = 3 KHz, sampling rate = 10.666 s^{-1}) (IDAC-4-USB, Syntech, Germany) and analyzed in the computer (Autospike v.3.9, Syntech, Germany). The setup was shielded by a Faraday cage.

2.4 Stimuli and stimulation

A total of 18 compounds (Table 1) were used in single sensillum recordings. These include kairomonal compounds (host-plant volatiles and scolytid beetle pheromone components) that attract *M. galloprovincialis* (Pajares et al., 2004), the male-produced aggregation pheromone of *M. galloprovincialis* (Pajares et al., 2010), and volatile

components of smoke released by burning wood (Schütz et al., 1992; Hall et al., unpublished), since there is circumstantial evidence that *M. galloprovincialis* is attracted to burnt trees.

For ORN characterization, compounds were tested at 10 µg, and for dose-response curves were tested at 1 ng to 10 µg in decadic steps. Dilutions were maintained at -20 ºC until used. Stimuli cartridges were prepared by applying 1 µl of solution onto a 20 x 1 mm piece of filter paper (#1, Whatman International Ltd, England) which was introduced into a 100 µl glass micropipette (1.2 mm internal diameter, Blaubrand® Intramark, Germany). Both filter paper and micropipeete were precleaned with *n*-hexane. Blank stimuli were prepared with 1 µl of the solvents used to make the dilutions, *n*-hexane and methylene chloride. Stimulus pipettes were prepared daily and kept in individual odor-clean glass tubes with teflon-lined screw caps. To avoid loss of stimulus, the base and the tip of micropitetes were sealed with parafilm until puffed.

A stimulus controller unit (CS-55, Syntech, Germany) produced a constant charcoal-filtered and humidified room-air flow of 0.5 l/min at 10 mm from the antenna (velocity at exit = 0.4 m/s). The stimulus pipette was placed at 5 mm from the contact point of sensillum and electrode, and a puff of charcoal-filtered room air sent stimulus-loaded air from the pipette to the preparation for 0.2 s (velocity at exit = 2.9 m/s). During the puff flow the continuous flow was stopped. The air around the preparation was constantly renewed with an exhaust to minimize contamination.

Every session started and finished with blank stimuli (*n*-hexane and CH_2Cl_2) and the other compounds were tested in a randomized sequence, preventing adaptation of

receptors by leaving 60 s between puffs. In all, 31 and 28 sensilla from 5 males and 7 females respectively were stimulated. For dose-response curves a different set of 21 and 19 sensilla from 2 males and 3 females was used. First we identified neurons with high sensitivity to sex pheromone, *cis*-verbenol or one of two blends, each containing half of the remaining compounds. When a sensitive cell was identified, it was stimulated with the 5 doses of the blend compounds.

2.5 Spike and statistical analyses

When two spike amplitudes were detected in the same sensillum, they were considered as different ORNs. To calculate relative spike frequency, the number of action potentials (spikes) during 1 s immediately prior to stimulation was subtracted from the number of spikes during 1 s following stimulation. Hierarchical cluster analysis with Ward's minimum variance method was used to group cells according to their response pattern. We observed considerable difference in latency (i.e., the time elapsed between stimulation and the ORN response) among compounds, so we analyzed this parameter for those compounds that produced clearly distinguishable excitations on a minimum of 7 ORNs. Then a general linear model (GLM) (Crawley, 2007) was fitted to compare latencies among compounds, and differences between means were tested for significance by Tukey's honestly significant difference test. Statistical analyses were performed under the statistical programming environment and language R, version 2.11.1 (R Core Team, 2012).

3. Results

3.1 Morphology

M. galloprovincialis shows the typical antennal sexual dimorphism of the *Monochamus* genus, in which male antennae are almost twice as long, in relation to the body, than those of females. Antennae have two basal segments, scape and pedicel, and nine flagellomers which get progressively smaller towards the apical segment, which is longer than the preceding one. All the sensilla types described here have already been characterized by SEM and TEM in two other *Monochamus* species (Dyer and Seabrook, 1975), providing valuable information about their possible function, which will be used in here. In both sexes of *M. galloprovincialis*, the most conspicuous sensilla on the antennae were the "stout sensilla chaetica" (*sensu* Dyer and Seabrook, 1975) (Fig. 1A). As their name suggests, these are large and solid sensilla, 58.95 ± 1.5 μm long, and 6.93 ± 0.53 μm wide at the base (mean ± SEM, N = 10), and they increased in numbers towards the distal end of the antenna (Table S1). In males they became gradually thicker and laid flatter on the surface towards the dorsal side of the antennae (Fig. 1A), receiving the name of "male peg sensilla chaetica" (Dyer and Seabrook, 1975). These were 40.98 ± 1.46 μm long and 15.15 ± 1.71 μm wide at the base (N = 5, only males).

On the latero-ventral surface of segments 2 to 8 there were two sensory fields, one in the proximal half consisting of a groove in the cuticle (Fig. 1B), and another located in the distal half and shaped as a plate area (Fig. 1C and D). In the first segment only the distal sensory field was present. In both sexes these areas were carpeted with hundreds of small sensilla which, following Dyer and Seabrook (1975), are described as

sensilla basiconica. Two subtypes were distinguished within these sensilla basiconica, one being more cylindrical (15.47 ± 1.16 μm in length and 2.87 ± 0.15 μm in diameter, $N = 5$), and the other one more flattened (12.04 ± 0.75 μm in length and 3.93 ± 0.24 μm in diameter, $N = 10$), with the first being relatively more abundant than the second (Fig. 1E). A third type of sensilla, probably sensilla trichoidea according to the similarity of those described by Dyer and Seabrook (1975), was found in small numbers, increasing in abundance towards the distal end of the antenna (Table S1, Fig. 1F). They presented longitudinal fluting and were 45.36 ± 1.7 μm in length and 4.23 ± 0.16 μm in diameter ($N = 10$). Distribution and abundance of these sensilla types were not different between sexes (Table S1).

The distal end of each flagellomere was surrounded by a ring of large sensilla chaetica which overlapped with the following segment (Fig. S2a). They were 189.87 ± 20.55 μm in length and 10.51 ± 0.40 μm in diameter near the base. ORNs in these sensilla fired action potentials in response to movement, so they probably act as proprioceptors that indicate antennal segment position. Long sensilla chaetica (Fig. S2b) were found in so small numbers that they almost never fell in our sampling area, so their abundance is not reported. They were 158.35 ± 5.79 μm in length and 5.02 ± 0.21 μm in diameter (n = 4). Two dome-shaped organs were observed in our entire sensillar sampling, both of them inside the sensory fields, and they consisted on a small vault of about 2.5 μm in diameter (Fig. S2c). Gland pores clustered around stout sensilla chaetica (Fig. S2d), as described by Dyer and Seabrook (1975).

3.2 Single sensillum recordings

No action potentials were obtained from the abundant stout/male peg sensilla chaetica. However, electrophysiological contact with neurons within sensilla basiconica were relatively easy to achieve, so we focused our sampling on these sensilla. Fifty of the 60 sensilla (83%) contained a single ORN and the remaining 17% contained two ORNs which could be separated by their differences in spike amplitude (Fig. 2a). A diversity of electrophysiological responses was obtained, including both excitations and inhibitions with maximum values to +97 and –20 spikes s^{-1}, respectively (Fig. S3). Some cells could respond with excitation to one compound and with inhibition to another (e.g., females 2S and 25S, Fig. S3).

Hierarchical cluster analysis grouped cells in distinct functional types. Because the groups were similar in males and females (data not shown), we combined ORNs from both sexes in a single and larger dataset to gain more resolution. Six roughly uniform ORN types were obtained this way (Figs. S3 and S4), and averaged responses of each type to all the test compounds are shown in Fig. 3. The largest group of cells (13 male and 10 female, 33 % of the total) did not respond to any stimuli and so were labeled as "Unresponsive". The next largest group (20 % of the ORNs, 3 from males and 11 from females) contained "Generalist" cells which showed heterogeneous, but relatively strong, responses to one or more compounds, but different for each cell. A group of 10 ORNs labelled "Smoke inhibited" (14% of the total, 4 males and 6 females) showed moderate but consistent inhibition by smoke compounds. A group of 8 cells (11% of the total, 3 male and 5 female) was, except for one cell, strongly excited by the pine volatile α-pinene and, depending on the cell, also responded to other host compounds (mainly (+)-camphene) and to bark beetle kairomone (mainly *cis*-verbenol) and smoke compounds (mainly 2-methoxyphenol and 4-methyl-2-methoxyphenol), so it

was labelled "α-pinene/generalist". A group of 9 cells (13 % of the total, 4 male and 5 female) responded strongly to the smoke compounds eugenol and 4-methyl-2-methoxyphenol, and were labelled as "Smoke-excited". Finally, a group of 6 cells (9 % of the total, 5 male and 1 female) responded very strongly and very specifically to the male-produced aggregation pheromone, so they were positively considered as "Pheromone" specialist cells.

Dose-response curves were obtained for some compounds (Fig. 4). Pheromone cells showed the typical sigmoidal-shape curve in the log(conc) scale (Byers, 2013) at the doses tested (0.1 to 10,000 ng), with no apparent leveling-off that would suggest saturation at the maximum concentration. Male and female pheromone ORNs showed similar intensity of response to pheromone ($F = 2.99$, $P = 0.085$, df = 1). Although only one female pheromone-specific ORN was found while screening odorants (Fig. S4) and only 9% of all the ORNs were pheromone-specific (Fig. 3), it was relatively easy to find pheromone-responding cells in males and females to make the dose-response curves, which indicates that these cells are relatively abundant. α-pinene/generalist cells were also relatively easy to contact and dose-response curves were made for 6 of them. These cells were not as sensitive as the pheromone cells, but there was a clear dose-response at the doses tested. Five smoke (eugenol) cells were recorded and showed a slightly different pattern from the other cells in that they responded very little to the 0.1 to 1,000 ng doses but peaked at the 10 µg dose (Fig. 4). Two more cells were tested with *cis*-verbenol. One showed a clear dose-response but the other did not. One cell was tested with ipsenol and another with ipsdienol and they clearly increased their response with an increase in concentration (Fig. 4).

Latency of response varied significantly among compounds ($F = 2.47$, $P = 0.005$, df = 13; Fig. S5 and illustrated in Fig. 2C). Although the same dose was used for all compounds, each has a different boiling point, and so it is likely that the number of molecules hitting the antenna varied. In moths, higher stimulus concentration result in shorter latencies (Jarriault et al., 2010), and this was the same in *M. galloprovincialis* when we compared the latency of response with the concentration of pheromone ($F = 20.31$, $P < 0.001$, df = 77). Therefore, it is possible that differences in latency reflected differences in compound quantity, in addition to, or instead of, the specific interaction of each compound with the olfactory receptor machinery (olfactory receptor protein, odor degrading enzymes, etc.). To address this possibility, we compared the intensity of the response with the boiling point of the compounds (pooling several ORNs) and found no relationship between these two variables ($F = 0.90$, $P = 0.345$, df = 167), which suggests that the volatility of the compounds does not fully explain differences in latency.

4. Discussion

4.1. Sensilla morphology

The sensilla assortment of *M. galloprovincialis* closely resembles that of its sister species *M. notatus* (Drury) and *M. scutellatus* (Say) described by Dyer and Seabrook (1975). The function of the conspicuous and abundant stout and male peg sensilla chaetica is unknown. We did not obtain action potentials from them, but Seabrook and Dyer (1975) report in the other two *Monochamus* species that some of these sensilla are innervated, and they suggest that probably serve as mechanoreceptors. Sensilla trichodea of *M. notatus* and *M. scutellatus* have several characteristics of contact chemoreceptors, such as a pore at the tip, which could well be used to sense the cuticular hydrocarbons which are so relevant in chemical communication of Cerambycids (Allison et al., 2004) including *M. galloprovincialis* (Ibeas et al., 2008). When manually bent, the distal sensilla chaetica of *M. galloprovincialis* responded with spike trains (data not shown), which demonstrated that these are mechanoreceptors. Other structures, such as the dome shaped sensilla and the glands associated with stout sensilla chaetica are similar to those described for *M. notatus* and *M. scutellatus* (Dyer and Seabrook, 1975) and they probably have a similar, but yet unknown, role in these three species.

Clearly, from an olfactory perspective, the most interesting sensilla type in *M. galloprovincialis*, and probably in other Cerambycids (Dyer and Seabrook, 1978), are the small sensilla basiconica located in the sensory fields. The two types of sensilla basiconica reported in here are similar in morphology to those described for *M. notatus*

and *M. scutellatus* (Dyer and Seabrook, 1975) and other Cerambycids, such as *Psacothea hilaris* (Pascoe) (Dai and Honda, 1990) and *Phoracanta semipunctata* Fab. (Lopes et al., 2002). In *M. galloprovincialis* they are confined to two sensory fields in each flagellum, similar to *M. notatus* and *M. scutellatus* (Dyer and Seabrook, 1975). As in these two species, in *M. galloprovincialis* there was no remarkable sexual dimorphism with respect to the distribution of sensilla basiconica and their abundance along the antenna, suggesting that these sensilla have a similar olfactory role in both sexes. This hypothesis is supported by the finding that both males and females are similarly attracted to host plant odor, bark beetle pheromones and *M. galloprovincialis* aggregation pheromone (Pajares et al., 2004; Ibeas et al., 2007; Pajares et al., 2010).

As with the North American *Monochamus* species (Allison et al., 2001; 2003; De Groot and Nott, 2004; Miller and Asaro, 2005), *M. galloprovincialis* adults of both sexes locate suitable host trees for breeding by following pine volatiles and sex pheromones of sympatric bark beetle species (Pajares et al., 2004; Ibeas et al., 2007), which correlates with the presence of ORNs tuned to these compounds. Host monoterpenes are defensive compounds located in trunk and leaves of woody plants. Some of these compounds are released in large quantities by stressed or recently damaged tissues, which signal optimal host conditions to potential predators. Among the monoterpenes, α-pinene is a major component in local pine species (Santos et al., 2006) and seems to be a key component for *M. galloprovincialis* to locate potential hosts (Ibeas et al., 2007; Pajares et al. 2010), which is consistent with ORNs responses found in our work. We also found ORN responses to β-pinene and 3-carene, which are also abundant monoterpenes in typical pine species of the Iberian Peninsula (Santos et al., 2006). Field bioassays have shown that α-pinene, 3-carene and β-pinene, in this

order, resulted the best pheromone synergists to *M. galloprovincialis* of 7 pine terpenes tested (Álvarez et al, unpublished). On the contrary, (Santos et al., 2006) reported myrcene as a relatively abundant monoterpene, but we did not observe much response to this compound.

All four bark beetle pheromone components tested in SSR are emitted by the European *Ips* species infesting pines: *Ips sexdentatus* (Boerner), *Ips acuminatus* (Gyllenhall), *Ips mannsfeldii* (Watchl) and *Ips (Orthotomicus) erosus* (Wollaston) (Kohnle et al. 1988, 1993). Although these are generally secondary species breeding in stressed, fallen or dying trees, they can kill healthy trees under favourable conditions. Since the colonization flights of these species overlap widely with that of *M. galloprovincialis* during the summer, it has been suggested that it is advantageous for the pine sawyers to respond to the pheromonal signals released by these secondary bark beetle species (Pajares et al., 2004). In North America, *cis*-verbenol does not attract *Monochamus* species (Allison et al. 2001, 2003; De Groot and Nott 2004), while the effect of ipsdienol varies according to the *Monochamus* species, being attractive to *M. titillator* (Miller and Asaro 2005), *M. clamator* and *M. scutellatus* (Allison et al. 2003), and unattractive to *M. scutellatus* and *M. mutator* (De Groot and Nott 2004). Field tests with *M. galloprovincialis* have shown that ipsdienol and *cis*-verbenol are behaviorally active, although less active than ipsenol and 2-methyl-3-buten-2-ol, and these two compounds constitute the kairomonal basis of commercial lures developed for *M. galloprovincialis* (Ibeas et al. 2007). Despite the behavioral importance of the bark beetle kairomones for *M. galloprovincialis*, we have not identified an ORN "type" that preferentially responds to them, if not for the "α-pinene/generalist" cells that in addition of responding to α-pinene and smoke compounds, also responded to *cis*-verbenol.

However, the two isolated dose-response curves to ipsenol and ipsdienol showed that at least some ORNs have high sensitivity to these compounds.

Forest fires debilitate or kill trees, and some xylophagous insects, mainly bark beetles, benefit from attacking weakened hosts, so it is to their advantage to locate burnt trees (Allison et al., 2004; Schwilk et al., 2006). Beetles detect recently burnt trees in two ways, by using infrared sensors located on their cuticle (Schmitz et al., 1997), and by smelling smoke compounds (Schütz et al., 1999). Although not proved experimentally, there is some evidence that *Monochamus* beetles are attracted to burnt trees (Parmelee, 1941; Ross, 1960, 1966). They have been reported on burnt hosts (Markalas 1997), and larvae are relatively easy to find in burnt pine stands (personal observation). Schütz et al. (1999) used the antenna of *Melanophila acuminata* Eschscholtz beetles to identify, by means of GC-EAD, the smoke compounds that may attract them to burnt trees, and these were mainly phenolic compounds similar to the ones we have tested, with 2-methoxyphenol (guaiacol) producing the strongest responses. In *M. galloprovincialis* three groups of neurons responded to smoke compounds as determined by hierarchical cluster analysis. The "smoke-excited" group was fairly specific in its response to smoke odorants, especially to eugenol and to 4-methyl-2-methoxyphenol. A second group of cells, the "α-pinene/generalists", was not so smoke-specific, as it responded to α-pinene *cis*-verbenol and other kairomones, in addition to the smoke compounds 2-methoxyphenol and 4-methyl-2-methoxyphenol. A third group of cells was slightly inhibited by smoke compounds. Therefore, *M. galloprovincialis* seems to be equipped with a refined sensory system dedicated to the detection smoke-related volatiles.

In *M. galloprovincialis* the aggregation pheromone, 2-(undecyloxy)-ethanol, is released by males, elicits EAG responses in both sexes, and attracts both males and females in the field (Pajares et al, 2010). The same molecule is also the aggregation pheromone of *M. alternatus* (Hope) (Teale et al., 2011), *M. carolinensis* (Olivier) and *M. titillator* (Fabricius) (Allison et al., 2012), *M. scutellatus* (Fierke et al. 2012), and *M. sutor* (L.) (Pajares et al., 2013). In addition, it has also been shown to be a likely pheromone component for *M. clamator* (LeConte) and *M. obtusus* Casey (Macias-Samano et al. 2012), and *M. notatus* (Fierke et al. 2012). The presence of 2-(undecyloxy)-ethanol-specific ORNs in both sexes confirms the ecological importance of this attractant for *M. galloprovincialis*. Although only one pheromone-specific ORN was found in females, as compared with 5 in males, these cells were relatively easy to find to make the dose-response curves, so we suspect that these cells are relatively common in both sexes. Given the importance of this compound for other *Monochamus* species, it is very likely that they also have specific receptors for it, and most likely in the basiconic sensilla of the plate areas.

We conclude that the antennal sensilla of *M. galloprovincialis* are similar morphologically and electrophysiologically to those described for other species in the same genus. Sensilla basiconica are chemoreceptors that respond to odors from host plants, bark beetles, smoke volatiles and aggregation pheromone. A few of the ORNs could be considered specific of pheromone or smoke compounds, and a large proportion are generalist or unresponsive, which suggest that many semiochemicals relevant to this species remain still unknown. There are no marked differences between sexes in relation to their receptors, so it is likely that behavioral activity depends on central integration of different stimulus. The presence of smoke-detector ORNs in *M.*

galloprovincialis supports the hypothesis that they use smoke odor to locate suitable mating and oviposition sites.

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Table 1: Synthetic compounds used in the bioassays.

Compound	Abbr.	BP (ºC)	Solvent	Purity	Source
Aggregation pheromone					
2-undecyloxy-1-ethanol	PHER	390	n-hexane	98%	NRI
Host-plant volatiles					
(+)-Limonene	LIMO	176	n-hexane	≥93%	SEDQ
(+)-Camphene	CAM	159	n-hexane	≥90%	SEDQ
p-Cymene	PCYM	177	n-hexane	≥97%	SEDQ
α−Pinene	APIN	155	n-hexane	≥97%	SEDQ
β−Myrcene	MYR	167	n-hexane	≥90%	SEDQ
β−Pinene	BPIN	155	n-hexane	≥97%	SEDQ
3-Carene	3CAR	168.5	n-hexane	≥90%	SEDQ
Bark beetle pheromones					
2-Methyl-3-buten-2-ol	2M3B	98.5	n-hexane	≥98%	SEDQ
Cis-verbenol	CISV	214.9	CH_2Cl_2	97%	SEDQ
Ipsdienol	IPSD	233.59	n-hexane	93%	SEDQ
Ipsenol	IPSE	222.2	n-hexane	93%	SEDQ
Smoke volatiles					
2-Methoxyphenol	2M	205	CH_2Cl_2	≥98%	SA
4-Methyl-2-methoxyphenol	4M2M	221.5	CH_2Cl_2	≥98%	SA
4-Vinyl-2-methoxyphenol	4V2M	224	CH_2Cl_2	≥98%	SA
Eugenol (2-methoxy-4-allylphenol)	EUG	254	CH_2Cl_2	≥98%	SA
Iso eugenol (2-methoxy-4-propenylphenol)	IEUG	266	CH_2Cl_2	99%	SA
Vanillin (4-hydroxy-3-methoxybenzaldehyde)	VAN	285	CH_2Cl_2	≥97%	SA

BP, boiling point. NRI, Natural Resources Institute, Chatham Maritime, Kent, UK. SEDQ, Sociedad Española de Desarrollos Químicos, Barcelona, Spain. SA, Sigma-Aldrich, Gillingham, Dorset, UK.

Figure 1

Figure 1

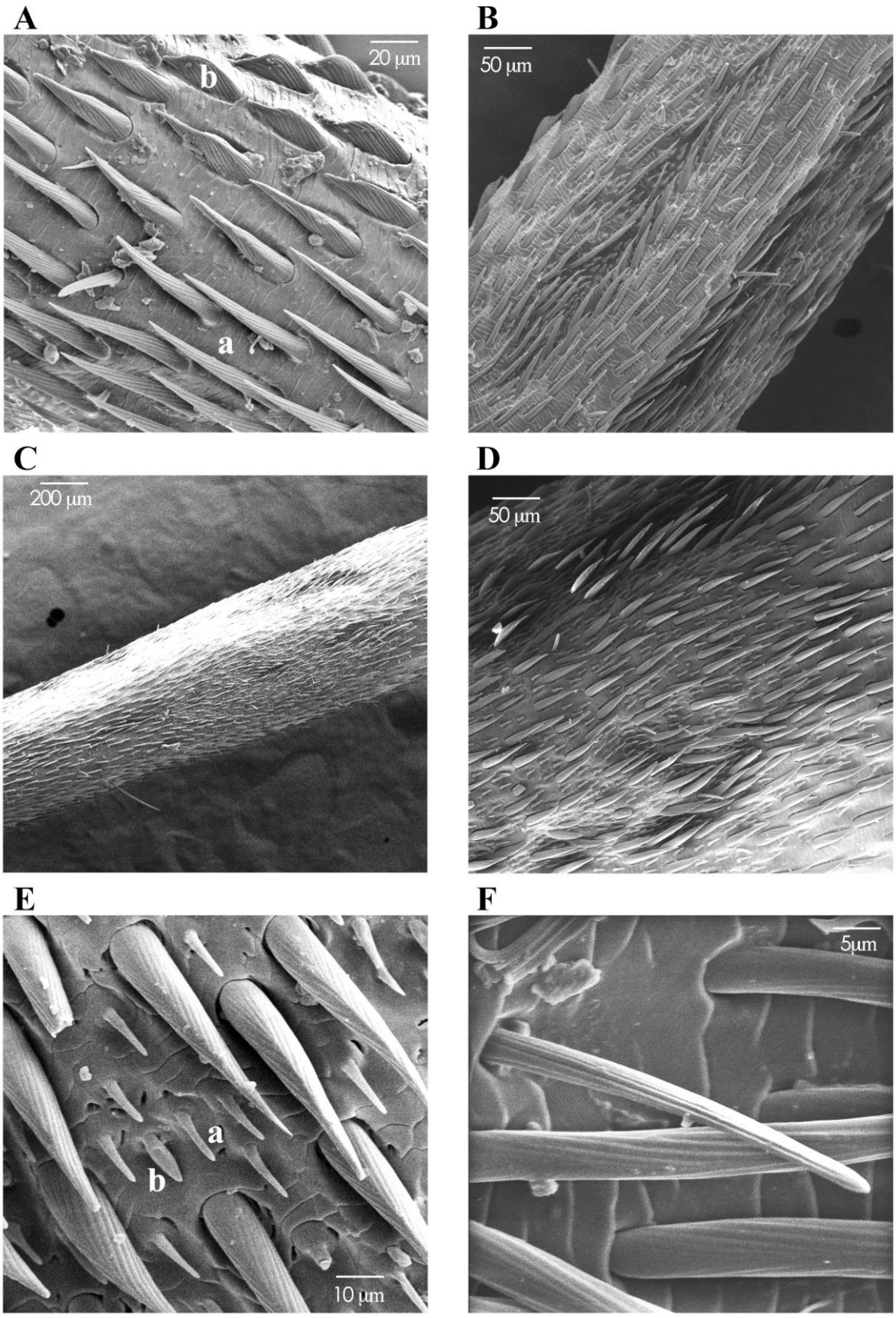

Figure 2

Figure 2

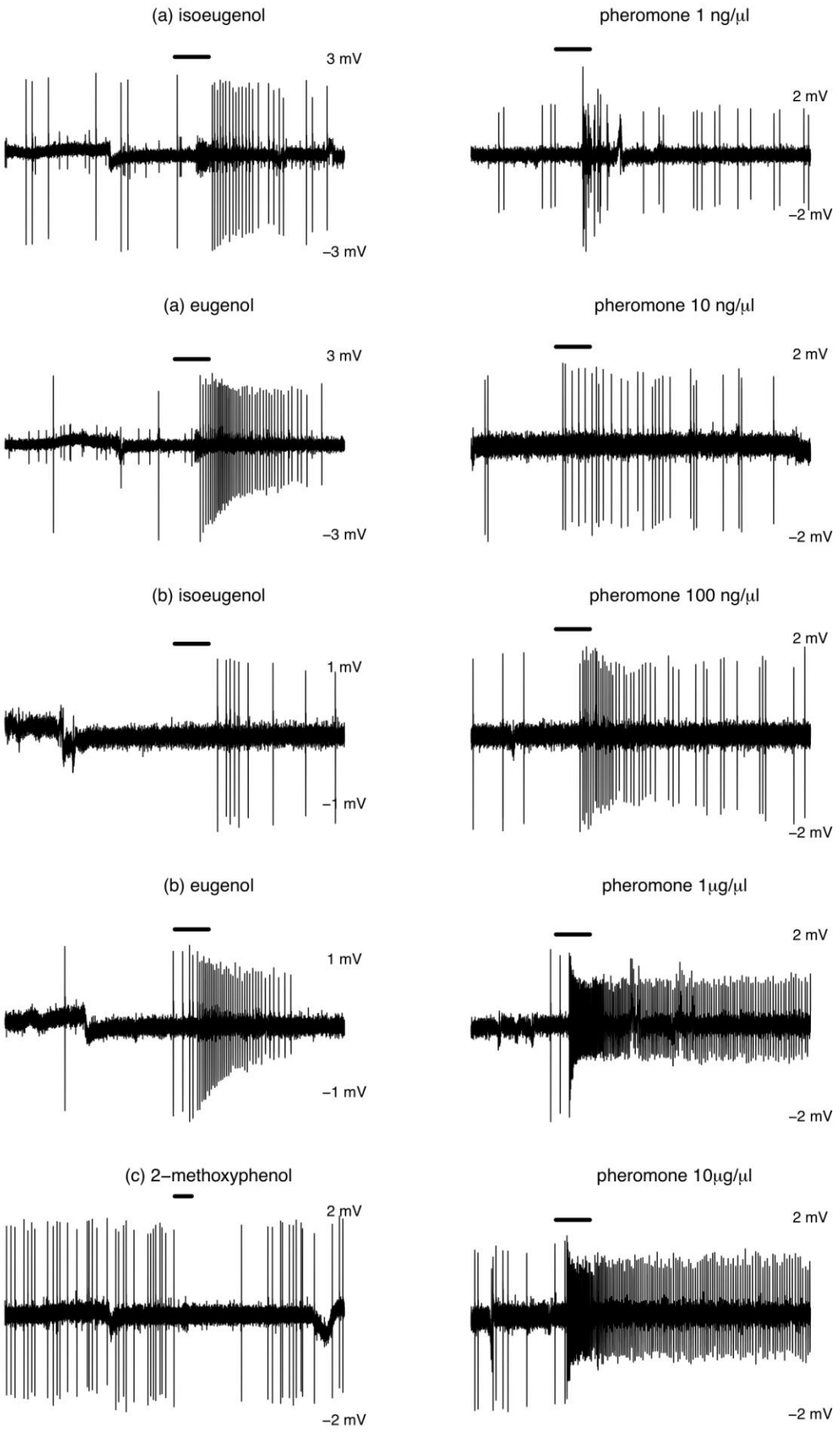

Figure 3

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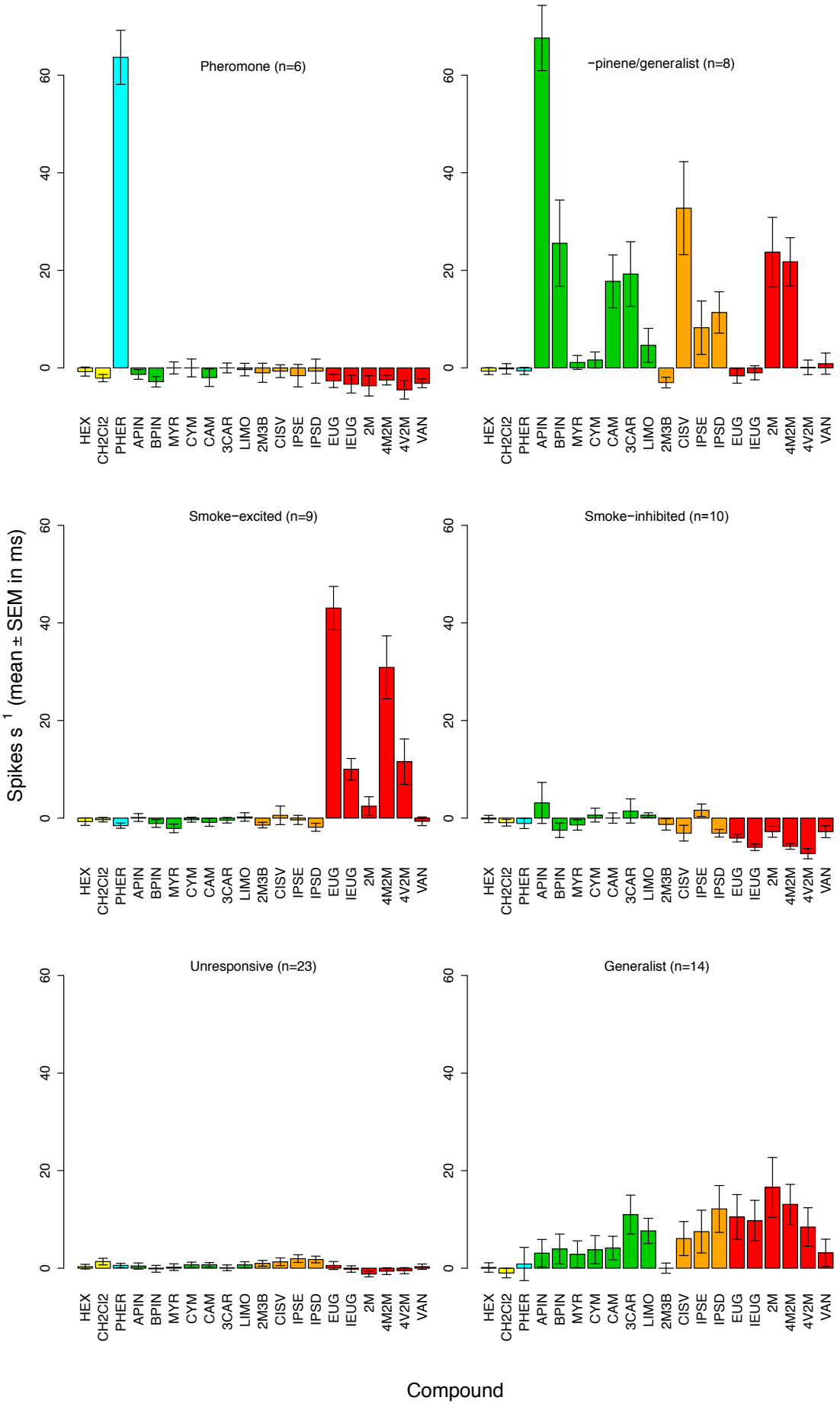

Figure 4

Figures

Figure 4:

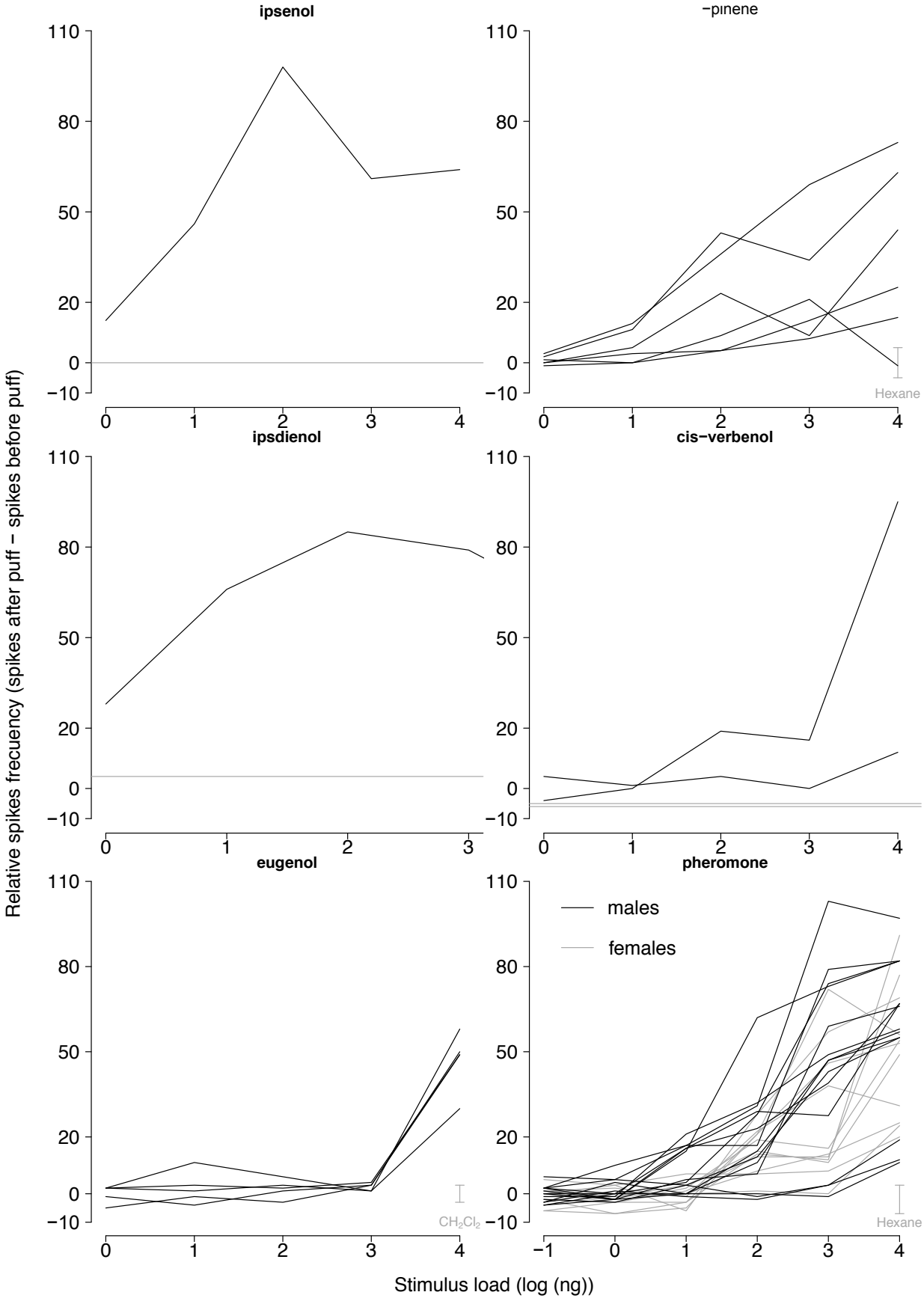

Figure captions

Fig. 1. Scanning electron micrographs of *M. galloprovincialis* antennae. (A) Stout sensilla chaetica (a) and male peg sensilla chaetica (b) (male). (B) Groove-shaped sensory field located at the proximal end of the flagellum (male). (C) Plate area sensory field located at the distal end of the flagellum (female). (D) Detail of plate area sensory field showing the small sensilla basiconica and the larger sensilla chaetica (male). (E) Cylindrical (a) and flattened (b) sensilla basiconica. (F) Probably sensilla trichoidea (female).

Fig. 2. Single sensillum recordings from sensilla basiconica in the plate areas. (a) Two cells in the same sensillum of a female, distinguishable by their action potential amplitude (the large one is about 6 mV, and the small one is about 1 mV). The large action potential cell is excited by isoeugenol (top) and by eugenol (bottom), and shows different latency to each one of them. The small action potential cell appears to be excited by isoeugenol. (b) A recording of a female sensillum with an isolated large neuron with very different response intensity and latency to isoeugenol (top) and eugenol (bottom). (c) Complete inhibition of response to 2-methoxyphenol in a female ORN. The right half of the figure shows a sequence of responses of a single male ORN to several doses of the sex pheromone, which illustrates both, the increased spike frequency and decreased latency as the concentration increases. Horizontal bar represents the 200 ms stimulation.

Fig. 3. Average response of ORN classes obtained from hierarchical cluster analysis (Fig. S4) to stimulation with 10 μg of test compounds. Colors indicate different categories of volatiles (yellow: solvents, blue: *M. galloprovincialis* aggregation pheromone, green: host monoterpenes, orange: bark beetle kairomones, and red: smoke volatiles; abbreviations as in Table 1).

Fig. 4. SSR doses-responses curves of *Monochamus galloprovincialis* ORNs to kairomone, smoke and aggregation pheromone. Each line represents a different ORN from a different sensillum. For some compounds only one ORN was recorded. Ipsenol, ipsdienol and eugenol were tested on males, and α-pinene and cis-verbenol were tested on females. Pheromone was tested on both sexes. Solvent response of individual ORNs is indicated as a horizontal grey line, or as a vertical line when there are many cells in the same plot.

Smoke, pheromone, and kairomone olfactory receptor neurons in males and females of the pine sawyer Monochamus galloprovincialis (Olivier) (Coleoptera: Cerambicydae)

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Supplementary figures and tables

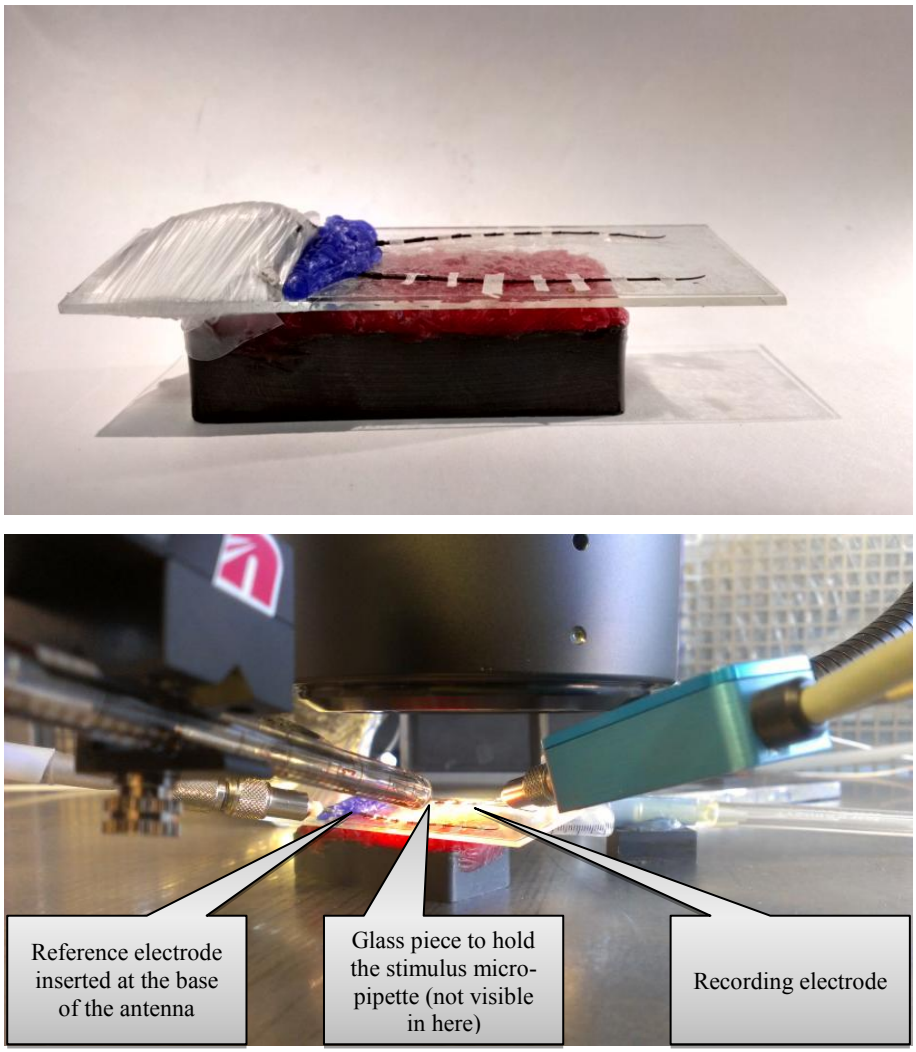

Figure S1. Insect preparation for SSR recordings. The body was immobilized on a microscope slide using paraffin plastic, the head was immobilized with dental wax and the antennae lay on double-sided sticky tape and are held with several strips of painter´s tape. The slide is fixed on a magnet using dental wax. After recordings, the insects were in returned to their rearing and resumed their normal behaviour containers apparently unharmed.

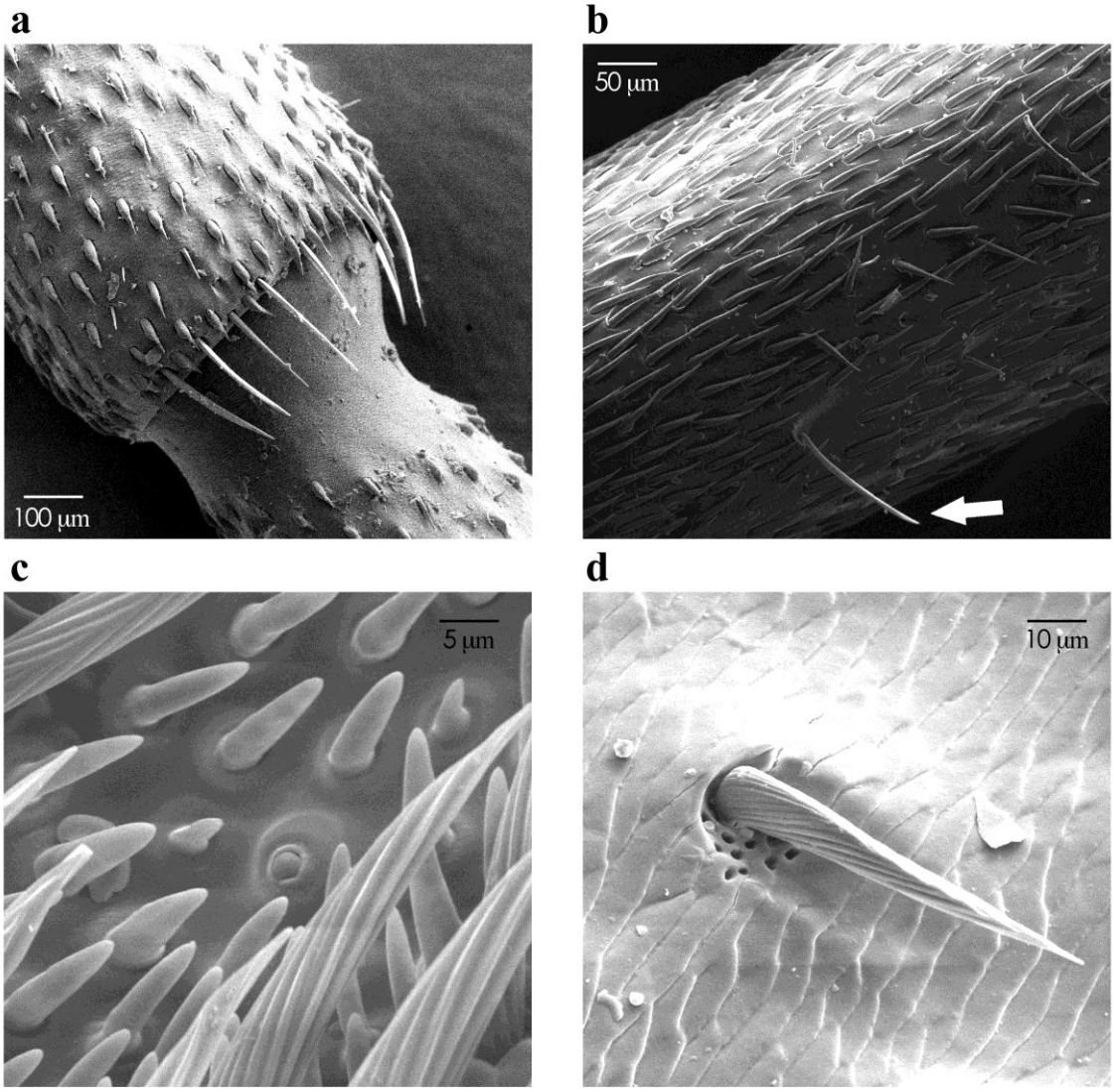

Figure S2. SEM pictures of *Monochamus galloprovinicalis* antenna. a) Distal end sensilla chaetica; b) Long sensilla chaetica (female); c) Dome shaped organ (male); d) Cluster of pores behind stout sensilla chaetica (male).

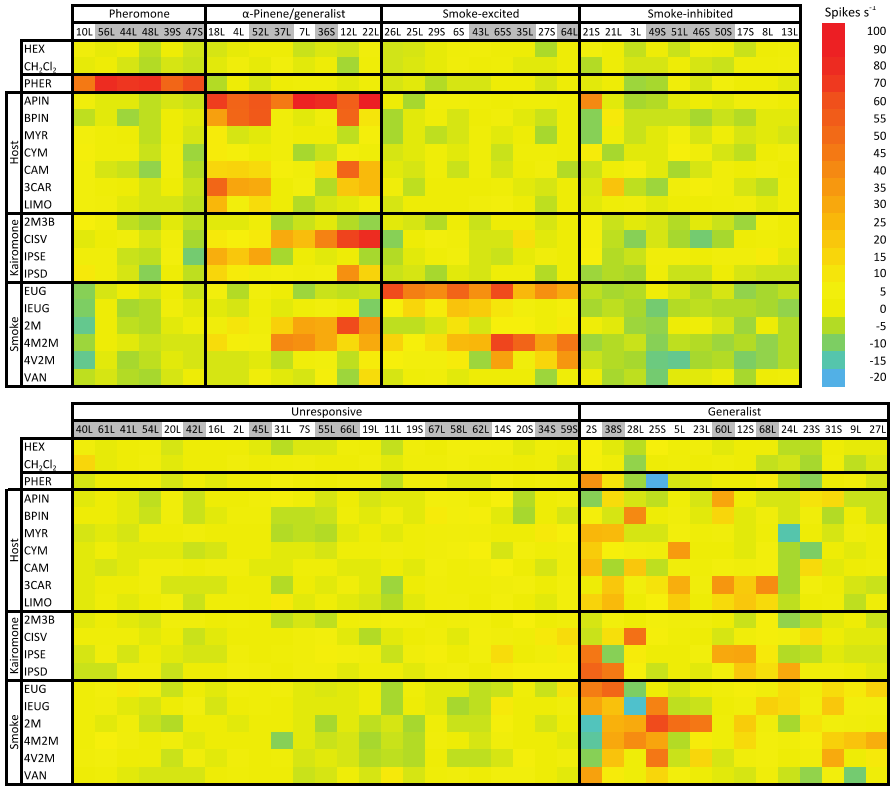

Figure S3. Response espectra of ORNs of *Monochamus galloprovincialis* to 10 µg of pheromone, kairomone and smoke odorants. Bar shows color code for inhibition (blue-green), no or minor response (yellow range), and moderate to large excitation (orange-red). Sensilla could house one or more (small [S] or large [L] action potential amplitude) cells. Female and male cells colored white and grey, respectively. Responses to the solvents (n-hexane or CH2Cl2) are also shown.

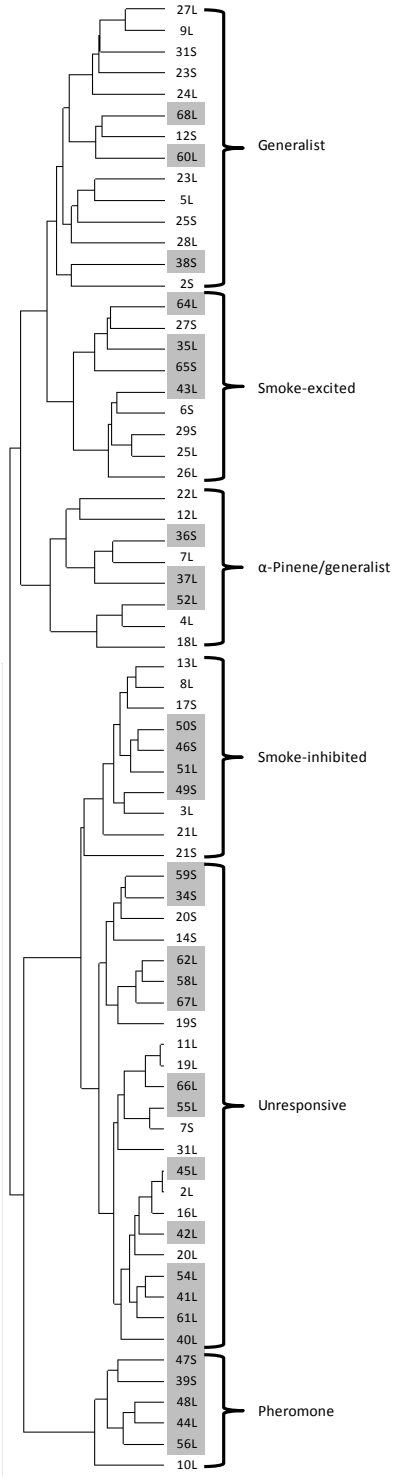

Figure S4. Dendrogram showing ORNs classes in *Monochamus galloprovincialis* males (empty cells) and females (grey cells). The dendrogram is based in a hierarchical cluster analysis.

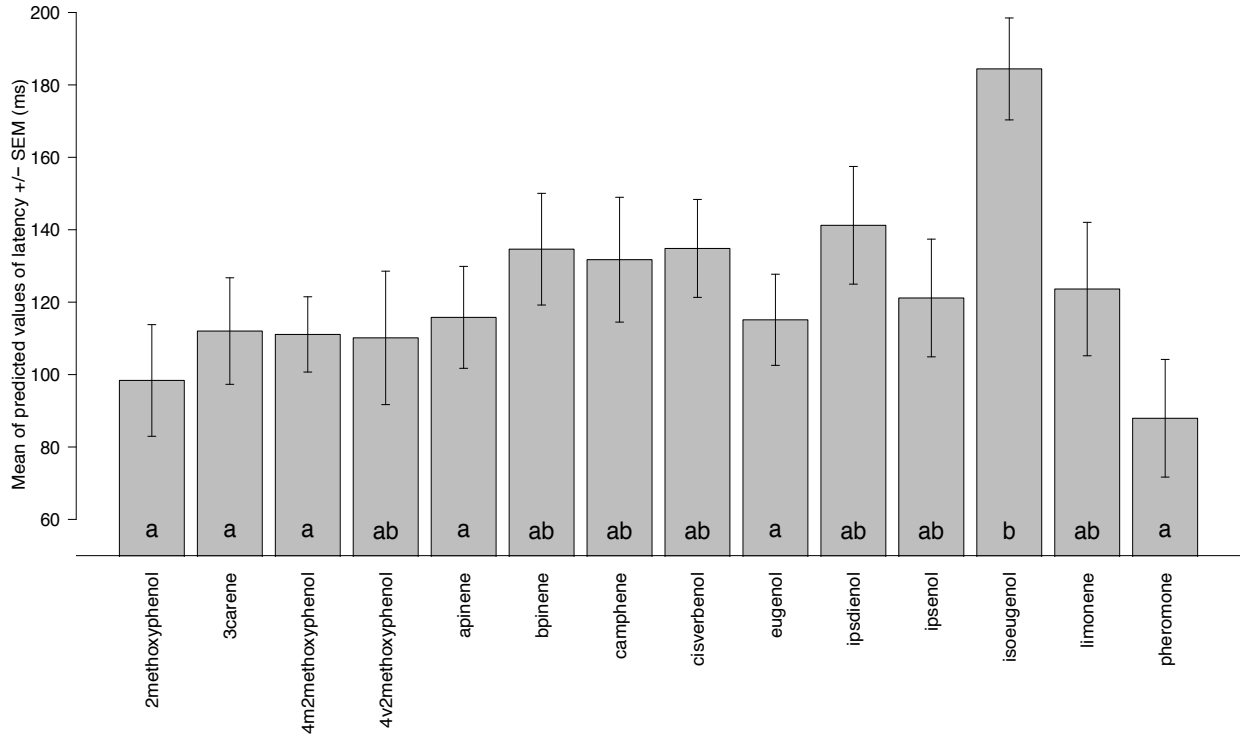

Figure S5. Mean (± SEM) latency of response of *Monochamus galloprovincialis* antennal ORNs to host, bark beetle, pheromone and smoke odorants. Different letters indicate significantly different latencies (Tukey's honestly significant difference test, $\alpha = 0.05$ after GLM). Only compounds with 7 or more replicates were used for comparison. The data shown in here are the predicted means and SEMs after GLM model analysis.

Table S1. Number of different sensilla types (mean ± SEM) sampled in 200 x 200 µm sections of segments 1, 3, 6 and distal, and in the scape and pedicel of *M. galloprovincialis* males and females

		Antennal segment					
	Sensillum type	Scape	Pedicel	1	3	6	Distal
Males	Stout chaetica	10.00 ± NA	13.75 ± 1.80	3.42 ± 1.15	9.92 ± 5.35	17.92 ± 6.22	24.44 ± 8.68
	Male peg	0.00 ± NA	0.00 ± 0.00	9.75 ± 2.08	10.83 ± 2.21	8.67 ± 2.14	18.67 ± 3.13
	Trichoidea	0.00 ± NA	0.00 ± 0.00	0.41 ± 0.19	0.83 ± 0.27	1.50 ± 0.31	3.11 ± 0.89
	Basiconica	0.00 ± NA	0.00 ± 0.00	5.25 ± 4.57	14.50 ± 9.28	24.33 ± 10.19	0.00 ± 0.00
Females	Stout chaetica	9.50 ±1.71	25.50 ±2.53	27.58 ± 1.32	43.25 ± 3.62	59.92 ± 6.65	75.67 ± 11.01
	Trichoidea	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.14	1.08 ± 0.43	2.58 ± 0.82	2.92 ± 1.07
	Basiconica	0.00 ± 0.00	0.00 ± 0.00	2.17 ± 2.17	16.67 ± 9.83	35.25 ± 12.20	26.17 ± 8.34